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ANEUPLOIDY TEST DEVELOPMENT: KINETOCHORE STAINING IN MAMMALIAN SYSTEMS

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16. ABSTRACT

The purpose of this project was to determine the feasibility of using human-derived antibodies against the chromosomal kinetochore region coupled with immunofluorescence staining as a method for evaluating the induction of aneuploidy in mammalian cells in vitro and in vivo. This technique was applied to (1) Chinese hamster cells (V79 cells), human fibroblasts, and peripheral lymphocytes interphase cells in vitro; (2) mouse bone marrow cells in vivo; and (3) mature human and rat sperm. Although kinetochore staining can be accomplished on a routine basis on mammalian interphase in vitro and in vivo cells, the technique does not appear to offer the staining intensity and/or persistence to allow for an accurate enumeration of the total genomic complement of a Chinese hamster, mouse or human cell. It was found that kinetochore structures are not visible in nature before or after techniques to cause sperm head enlargement. Kinetochore structures can, most likely, be detected in micronuclei.

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1. INTRODUCTION

The ability of chemicals to induce chromosomal malsegregation during cytokinesis in mammalian cells constitutes a serious human health hazard (Dellarco et al., 1985). Numerical chromosomal anomalies (i.e., aneuploidy, the loss or gain of one or more whole chromosomes from the chromosomal complement of a somatic or germ cell) are the major cause of early gestational spontaneous abortions in humans (Hook, 1985). In addition, there is increasing evidence that aneuploidy in somatic cells may play a role in the etiology of carcinogenesis (Barrett et al., 1985). While short-term tests for the detection of aneuploidy-inducing agents exist, few approaches using mammalian cells in culture, or more importantly, in the intact mammal, are available. The majority of mammalian assays involve direct cytogenetic analysis of the chromosomal complement of metaphase cells. The validity of this approach is frequently confounded by technical artifacts associated with the loss and, to a lesser extent, the gain of isolated chromosomes during slide preparation. Thus there is a need for additional mammalian cell oriented tests, especially those that are reliable, sensitive, and cost-effective.

The recent availability of human-derived antibodies against the chromosomal kinetochore region, as described by Brenner et al. (1981), coupled with the use of immunofluorescence staining techniques, suggest another approach for evaluating the induction of aneuploidy cells in vitro and in vivo. The purpose of this project was to determine the feasibility of using the kinetochore antibody staining technique as developed by B.R. Brinkley (Baylor Medical College, Houston, TX) (personal communication) to enumerate the number of chromosomes in (1) mammalian interphase somatic cells in vitro and somatic in vivo, (2) mature sperm, and (3) micronuclei in vitro and in vivo.

2. MATERIALS

Human scleroderma kinetochore antibodies (used at a titer of 1:200), goat antihuman IgG tagged with fluorescein (originally from Miles), and sheep antihuman tubulin antibodies were kindly provided by Dr. B.R. Brinkley. Formaldehyde (EM grade) was obtained from Tousimis Research Corp.; Trion-X100, poly-L-lysine (mw 40,000), propidium iodide, dithiothreotol, proteinase K, ethylene glycol, and pronase from Sigma; phytohemagglutinin, penicillin-streptomycin, L-glutamine, McCoy's 5A medium, Ham's F10 medium, fetal bovine serum from Gibco; and Giemsa from Harleco. Plastic ware was obtained from Corning. The cell lines used (GM 2345, V79, CHO) had been carried and/or remained frozen in the laboratory until use since 1976-1978. Peripheral blood and sperm samples were obtained from freely consenting donors. Mice (male) were of strain B6C3F1 obtained as weanlings from Frederick Cancer Research Center. Rats (male) were of strain Sprague-Dawley obtained from Taconic Farms. All animals were housed in stainless steel cages on corn cob bedding (Bed O'Cobs, J.R. Nielsen and Sons) at 22_0C, 50% relative humidity, 12 hr light:dark (0700 light), and provided food (Purina rodent chow) and water ad libitum. Information on methods and additional materials will be provided, as needed, in section 3.

3. EXPERIMENTAL PROCEDURES AND RESULTS

3.1. Mammalian Cells In Vitro Studies

The first series of experiments was directed at obtaining definitive kinetochore staining using the kinetochore antibodies and FITC-conjugated rabbit antihuman IgG provided by B.R. Brinkley. As suggested by Brinkley, the first studies used Chinese hamster cells *in vitro*. Chinese hamster cells

(V79) were cultured in Ham's F10 medium (10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine) to confluency, a cell suspension was obtained by trypsinization, and cells were seeded at low density ($10^4 10^5/cm^2$) onto sterilized No. 2 coverslips (11 x 22 mm) in petri dishes and allowed to attach for 24 hr. At the end of this time period, the petri dishes were flooded with prewarmed medium and the cells were allowed to proliferate until 60-80% confluency was attained (3 days). Colcemid (0.06 ug/ml) was added for the last three hours of culture. The coverslips were removed, rinsed well in phosphate buffered saline (PBS, pH 7.4), and the cells fixed with 3% formaldehyde (plus 1% DMSO in PBS) for 20 min. at room temperature. Following fixation, the coverslips were rinsed well in PBS, and placed in 0.5% Triton X100 (in PBS) for 90 sec. to permeabilize the cells. After rinsing the coverslips well in PBS again, the cells were exposed to the human kinetochore antibody (1:200 dilution, 37°C, humidified atmosphere) for 1 hr. After rinsing well and incubating for 10 min. in PBS, FITC-conjugated rabbit antihuman IgG was added to the coverslips for 45 min. at 37_0C. The coverslips were again rinsed well in PBS to remove non-complexed antibody. Cells were counter stained for DNA with porpidium iodide (PI, 10 gamma/ml, 15 min.), the coverslips were rinsed well in PBS, and the coverslips were mounted on a microscope slide using 9:1 glycerol:PBS (pH 8.5-9.0). Following this staining protocol, the slides were scanned at 1000x magnification for kinetochore-stained chromosomes in metaphase or interphase cells using a Zeiss epi-illuminated fluorescence microscope (100 W mercury source) equipped with a FITC dichroic mirror filter combination. Cells stained with sheep anti-human tubulin antibodies were used as a positive control for the FITC-conjugated second antibody reaction. This protocol was the basic staining protocol used throughout the following results unless indicated otherwise.

The first series of experiments were disappointing in that while tubulin and PI-stained interphase cells and metaphase chromosomes were clearly visible, no kinetochore structures were observed. Small dots, which could have been kinetochore structures, were observed occasionally but faded too rapidly for agreement among the observers to be reached.

After several failures to obtain kinetochore staining, Ms. L. Wibel (Brinkley's senior technician) visited our laboratory for several days to demonstrate the kinetochore staining technique. What became quickly obvious was that several of the staining steps had to be very closely monitored to avoid (1) dilution of the human kinetochore antibody to concentrations too low to detect kinetochores with the FITC-labeled second antibody, (2) light exposure between staining and microscopic examination, and/or (3) too much incidential light during microscopic evaluation. During Ms. Wibel's visit, several experiments were conducted using V79 cells which resulted in kinetochore staining. Following these modified procedures, the following experiments were attempted.

Following the basic protocol for growing V79 cells on coverslips and for immunofluorescence staining of kinetochore structures, several appropriately stained slides were scanned for clearly demarcated G_1 interphase cells exhibiting kinetochore staining. The number of kinetochores in individual cells was ascertained (Table 1). The normal chromosomal complement of V79 cells is 21 to 23 chromosomes. However, in no case did any of the three observers count the expected number of kinetochores among the 300 cells analyzed. Differences in mean number of kinetochores scored among observers were probably due to differences in visual acuity and, to some extent, differences in degree of magnification. Failure to count the normal chromosomal complement is believed to have been due to the thickness of the interphase cells on the coverslip and to the rapid fading of the FITC-dependent stain. The following experiments

Table 1. Kinetochore Counts in Interphase V79 Cells.

Scorer	Number Cells Analyzed*	Average Number of Kinetochores	Range
1	100	16	11-19
2	100	14	9-18
3	100	17	15-19

Normal complement is 21-23 chromosomes.

*All interphase cells were in G_1

were conducted in an attempt to obtain better resolution of kinetochore structures.

3.1.1. Human cells - It was considered possible that kinetochores in human cells may fluoresce more brightly and with greater persistence because the kinetochore antibodies were against human kinetochore regions. Thus, the attempt was made to visualize kinetochores in human cells using the techniques indicated above. In the first series of experiments involving human cells, human fibroblasts (GM 2345) were cultured in McCoy's 5A medium (10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine) on coverslips and processed as described for kinetochore staining. Kinetochore structures were observed, but a complete and accurate enumeration of these structures again proved impossible.

The same approach was also used with human peripheral blood lymphocytes stimulated to proliferate in vitro in McCoy's 5A medium (10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, 0.25 ml PHA-M). However, great difficulty was encountered in these studies due to the fact that very few cells remained fixed onto the coverslips after staining for kinetochore structure. Because the objective was ultimately to examine for kinetochores in cell suspensions obtained from in vivo tissues, the focus was then placed on achieving adequate kinetochore staining in cells in suspension while also increasing the reliability of kinetochore counting in interphase cells.

3.1.2. Poly-L-lysine - It was quickly discovered that mammalian cells (V79, human lymphocytes) would not remain attached uniformly to the coverslips during the processing for immunofluorescence staining. Consequently, the decision was made to coat coverslips with poly-L-lysine as a method for increasing the

frequency and stability of attachment of mammalian cells to the coverslips (Rajendra et al., 1980). Coverslips were coated with poly-L-lysine (Sigma, mw 40,000, 10 mg/ml) and allowed to dry. Two protocols were used to attach mammalian cells to poly-L-lysine-coated coverslips. The methods differed in whether gravity or a Cytospin was used to place the cells into contact with the coverslip. During this aspect of protocol development, acridine orange (0.125 mg/ml in phosphate buffer, pH 7.4 for 5 to 10 min, followed by incubation in phosphate buffer for 5 to 10 min) or Giemsa staining (4% plus 4% methanol in distilled water) was used to visualize the attached cells. Because fixation with formaldehyde was found to cause cell clumping in some situations, other fixation processes (i.e., absolute methanol, absolute ethanol) were used when necessary, depending upon the cell type (see section 5).

Both of the methods for placing cells in contact with poly-L-lysine-coated coverslips worked well (after extensive experimentation) for obtaining a density of attached cells great enough to be useful for microscopic evaluation. However, the cell density on the coverslips depended upon the original cell density, the fixation step, the cell type, the length of settling time for the normal-gravity dependent method, and the duration and speed of the Cytospin. Methods were developed which allowed for the routine reproducible attachment of mammalian cells (V79, human lymphocytes, mouse peripheral blood erythrocytes, mouse bone marrow, rat sperm, human sperm) to coverslips treated with poly-L-lysine. The major difficulties encountered with this method were a necessity to determine the optimal conditions for each cell type and the existence of an occasional coverslip on which the poly-L-lysine coating was too thick to allow adequate microscopic evaluation of the attached cells.

Using this approach, the attempt was again made to visualize kinetochores in human lymphocytes cultured from four donors. At first, kinetochore staining was not achieved until care was taken to keep the coverslips hydrated throughout the cell adhesion and staining procedures. Although kinetochore staining was ultimately achieved in human lymphocytes, an accurate determination of the correct number of kinetochores still proved impossible.

3.1.3. Attempts to manipulate cell thickness - To increase the ability to enumerate kinetochores in interphase somatic cells, the attempt was made to manipulate the three-dimensional structure of the attached cells. For these studies, the effect of hypotonic treatments on cell swelling was initially examined.

It was surmised that by enlarging cell size through hypotonic treatment, cell thickness might be decreased at the time of kinetochore staining. Therefore, V79 cells were grown to confluency in culture flasks and cells were collected by trypsinization. The cells were then exposed at 37°C for 10 min to various hypotonic strengths using potassium chloride (0, 1, 3, 6, 4, 7, 9.4, 18.7, 37.5, and 75.0 mM, and 100% PBS). After hypotonic treatment, the cell samples were either fixed in cold absolute ethanol for 10 min and spun onto poly-L-lysine-coated coverslips using a Cytospin (5 min., 1500 rpm) or fixed in cold absolute ethanol after the Cytospin step. Following attachment of the cells to the coverslips, the cells were stained with Giemsa (4% plus 4% methanol in dH_2O) and the frequency of attachment and the morphology of the cells were examined (Table 2). Based on these results, it was decided to evaluate immunofluorescence staining in V79 cells exposed to 25.0 mM KCl.

One other technical manipulation to decrease cell thickness was attempted. After exposing V79 cells to 25 mM KCl hypotonic, experiments were conducted to

Table 2. Hypotonicity Experiments

Sample Number	Hypotonic Treatment	ETOH fixative* before	ETOH fixative* after	Cell Morphology Quality**
1	PBS	+		+++
2	PBS		+	+++
3	75 mM KCl	+		+++
4	75 mM KCl		+	+++
5	37.5 mM KCl	+		+++
6	37.5 mM KCl		+	+++
7	18.7 mM KCl	+		++
8	18.7 mM KCl		+	+
9	9.4 mM KCl	+		+
10	9.4 mM KCl		+	0
11	4.7 mM KCl	+		0
12	4.7 mM KCl		+	0
13	2.6 mM KCl	+		0
14	2.6 mM KCl		+	0
15	1.3 mM KCl	+		0
16	1.3 mM KCl		+	0
17	dH_2O mM KCl	+		0
18	dH_2O mM KCl		+	0

* Fixative before or after pelleting onto coverslips

**V79 cells morphology Giemsa staining after

0 = Very poor morphology/no cells
+ = Poor morphology/poor cell number
++ = Adequate morphology/adequate cell number
+++ = Good morphology/good cell number

evaluate the effect of Cytospin speed (and thus g force) on cell morphology and number. A range of speeds (1500-5000 rpm) was attempted. It was found that with increasing speed, increasing amounts of cell debris were found on the coverslips. However, a two-step Cytospin, the first step at the usual 1500 rpm, followed by a short (2 min.) step at 4500 rpm, did appear to offer the chance for decreasing cell thickness without increased debris. Under these conditions, it was important to fix the cells after coverslip attachment.

Following these experiments, attempts were made several times to obtain good kinetochore staining in V79 cells exposed to 25 mM hypotonic KCl and Cytospun using a two-step process. However, no clear kinetochore staining occurred. The reasons for this lack of appropriate staining remain unresolved since attention was focused next on obtaining kinetochore staining in sperm and then in micronuclei.

4. SPERM STUDIES

One of the major goals of this research was to obtain kinetochore staining in mammalian sperm. It was believed that kinetochore enumeration in human sperm would allow for an unequivocal evaluation of the extent of nondisjunction in germ cell populations and would prove to be an extremely important tool in human monitoring studies. Consequently, a great deal of the emphasis of this study, once kinetochore structures were successfully visualized in mammalian interphase somatic cells *in vitro*, was placed on attempting to visualize kinetochore structures in mature human sperm.

The first series of experiments involved obtaining isolated human sperm free from the other cells and cell debris present in semen, attaching the cells to poly-L-lysine-coated coverslips, and staining for kinetochore structures as

described earlier. While relatively pure sperm suspensions were obtained and sperm attachment to poly-L-lysine-coated coverslips occurred, no kinetochore structures were ever visualized. As a possible improvement, several techniques for swelling sperm heads were investigated on the assumption that the lack of kinetochore staining could be due to the degree of chromosomal condensation that occurs in sperm.

4.1. Sperm Enlargement

Two techniques were used to cause sperm head enlargement. The first technique involved mixing the human semen sample with 100 mM Tris, 10 mM EDTA resuspending the sperm in 100 mM Tris, 10 mM EDTA, (PH 7.4); washing the cell suspension twice with 50 mM Tris, 5 mM EDTA; resuspending the spern in 100 mM Tris, 10 mM EDTA, 1% SDS at room temperature, adding 0.1 M diothiothreotol and incubating the sperm for 10 min followed by treatment with proteinase K for 2 hr at 45°C. Proteolysis is stopped by fixing with absolute methanol. The second technique involved washing sperm twice with Crebs Ringer's phosphate solution, incubating the sperm in Ringer's solution for 10 min. at 37°C, and then treating the sperm with 1% SDS for a variable length of time. Following extensive manipulations to optimize the sperm enlargement conditions, a good recovery of intact, tailless sperm enlarged some 2-4 times was reproducible. However, discrete kinetochore structures stained by immunofluoresence were not observed in any experiment following these two enlargement protocols. Under some circumstances, a band of FITC-stained material about one-third of the sperm head length from the tip of the sperm was observed.

To verify that these staining problems were specific to mature sperm, the feasibility of staining kinetochores in immature germ cells and mature sperm of Sprague-Dawley rats was examined. The right testis and vas were removed from

male Sprague-Dawley rats. The tunica of the testis was split and tubules were squeezed out with tweezers. The cells were minced and collected in PBS, treated with 750 mM KCl for 20 min., and fixed with either 3% formaldehyde or 100% absolute ethanol. Following fixation, much of the clumped material was removed by differential centrifugation and the remaining cell suspension was spun onto poly-L-lysine-coated coverslips. Staining cells in this material for kinetochores was successful. However, mature rat sperm, either with or without treatment to increase their size, failed to exhibit FITC-dependent kinetochore structures, suggesting that the failure to observe kinetochores in mature human sperm was a valid finding. Subsequently, B.R. Brinkley (personal communication) corroborated this lack of success with mature sperm and the observation of occasional FITC-stained bands in sperm sperm heads. The conclusion was that either the kinetochore structures in mature sperm have lost their antigenic determinants, or that they are not accessible to the antibody. Enlargement of sperm heads by the methods used either did not make the kinetochores more accessible or destroyed their antigenic determinants.

5. MICRONUCLEI IN MAMMALIAN CELLS IN VIVO AND IN VITRO STUDIES

Several experiments were conducted as part of the micronuclei experiments (see below) to examine whether kinetochore structures in mouse bone marrow cells could be obtained. Male B6C3F1 mice were killed by cervical dislocation and bone marrow was flushed from their femurs using PBS. The cells were pelleted by centrifugation and were then hypotonically treated (75 mM KCl, 37°C, 25 min), spun onto poly-L-lysine-coated coverslips, and fixed using 100% ethanol. Kinetochore structures were successfully stained after several technical manipulations to optimize the collection and coverslip preparation steps. Enumeration of kinetochore structures in individual interphase cells was not attempted.

It was decided late in the project that because the enumeration of kinetochores in mature sperm was not feasible with the present techniques and that the enumeration of kinetochores in somatic cells (at least those of Chinese hamsters, mice, or humans) in vivo or in vitro was technically too difficult, the evaluation of kinetochores in micronuclei (MN) could prove to be very useful. The presence of a kinetochore in a MN would be considered sufficient evidence that the MN had been induced by chromosomal nondisjunction and not through the formation of acentric fragments (i.e., lacking a kinetochore). Thus, kinetochore-bearing MN could be used to identify aneuploid-inducing agents. However, it was realized that the lack of a kinetochore was not sufficient evidence that an aneuploidy-related event had not been involved since inactivation of the kinetochore or loss of its antigenic determinants could result in the apparent absence of a kinetochore. It was hoped that kinetochore scoring would prove to be easy in MN since individual kinetochores did not have to be enumerated. Rather, what was important was whether any kinetochore staining was or was not present.

Because Dr. R.R. Tice's laboratory used the in vivo mouse peripheral blood micronucleus assay on a routine basis, the initial investigations focused on attempting to identify MN in mouse micronucleated peripheral blood erythrocytes. Great difficulty was encountered initially in developing the techniques for obtaining, in a reproducible manner, smears of peripheral blood suitable for the kinetochore staining process, while keeping a manageable cell density and morphology. In brief, after obtaining peripheral blood by retro-orbital bleeding, modifications to capillary tubes were made using the following experimental parameters in a series of experiments to optimize conditions for kinetochore staining: blood conditions (undiluted; diluted with PBS at 1:1, 1:2, 1:5,

1:10, 1:20; diluted with fetal bovine serum at 1:1, 1:2, 1:5, 1:10, 1:20), fixative (3% formaldehyde + 1% DMSO, 100% methanol, 100% ethanol), fixation time (before or after coverslip preparation), and Cytospin conditions (gravity, 1500, 3000 rpm x variable times). During these experiments, all coverslips were stained with either acridine orange or Giemsa (as described above) for examination of cell density and morphology. Several conditions were found to optimize the preparation of the coverslips for immunofluorescence staining, including the use of a cold 100% methanol fixation step after the cells adhered to poly-L-lysine, the use of a 1:1 dilution with fetal bovine serum, and the attachment to coverslips by gravity (37°C humidified, 15 min.). When coverslips of peripheral blood cells prepared by this method were processed for kinetochore staining, it was quickly discovered that the Triton X100 incubation used to permeabilize cells to the antibodies worked well for blood lymphocytes, but was often too destructive to erythrocytes. Subsequently, methods (less Triton X100, no Triton X100) were attempted which appeared to result in a lack of erythrocyte destruction. However, it was also discovered that it was extremely difficult to find micronucleated erythrocytes using the propidium iodide DNA staining technique. This led to several experiments using rhodamine-conjugated antihuman IgG and Hoechst 33258 DNA staining, which provided fluorescence spectra patterns that would not overlap, but might be better suited for visualizing MN. These different staining methods were used with peripheral blood obtained from mice that had been treated with colchicine (0.5 mg/kg, twice weekly) for 2 months. While the frequency of micronucleated erythrocytes was increased approximately sixfold in these animals, it was still difficult to clearly identify whether kinetochore staining was achieved in any of the few observable MN. At this time, efforts were turned toward achieving MN <u>in vitro</u> using a more simple system.

In the in vitro studies, the decision was first made to maximize the yield of MN in the Chinese hamster cell line CHO. CHO cells were cultured as described above for V79 cells. Cells were harvested at confluency and seeded onto sterile coverslips. In the first series of experiments, the optimal conditions were examined for inducing MN by Colcemid, a well-known aneuploidy inducer (Table 3). The investigators examined the effects of initial cell density (0.5, 2.5, 5 x 10^4 cells/cm^2), Colcemid concentration (0.01, 0.001, 0.0001 mg/ml), and termination time (24, 36, 48 hr) on MN frequency and cell density. Based on these results, another series of experiments using Colcemid at concentrations of 0.0001, 0.00025, and 0.0005 mg/ml, and initial seeding densities of 6 x 10^3 and 1.2 x 10^4 cells/cm^2 were conducted. It was decided that the optimal conditions for Colcemid-induced MN formation were about 0.0005 mg/mL Colcemid, 5 x 10^3 cells/cm^2 seeding density, and a variable incubation time. Coverslips that were 50% to 80% confluent were collected using visual inspection by phase content microscopy (generally about 3 days).

Additional experiments were conducted to induce MN in CHO cells by X-rays. CHO cells collected as described were exposed to 25, 50, and 100 rads of X-ray (250 Kvp, 30 mA), seeded at 1 x 10^4 cells/cm^2, and examined for the MN frequencies upon reaching near confluency. Under these conditions, 100 rads appeared to provide the maximum induction of MN without excessive cell toxicity.

Experiments were conducted to examine Colcemid- and X-ray-induced MN for kinetochores using the standard immunofluorescence technique as described above. A double blind study design was used and the X-ray-induced MN cells served as a putative negative control. The results of the analysis, while very suggestive, were not unequivocal. Difficulties in kinetochore staining occurred as evidenced by the lack of strong kinetochore staining interphase

Table 3. Induction of MN by Colcemid

A.

Colcemid (mg/ml)	Per Cent Cells with MN Termination Time 24 HR I	24 HR II	24 HR III	36 HR I	36 HR II	36 HR III	48 HR I	48 HR II	48 HR III
.01	1	0	1	2	1	2	3	4	3
.001	5	3	3	4	4	3	6	4	2
.0001	10	9	10	15	18	19	19	11	15
0	0	1	0	0	0	0	1	1	0

I = .5 x 10^4 cells/cm^2
II = 2.5 x 10^4 cells/cm^2
III = 5.0 x 10^4 cells/cm^2

100 interphase cells scored per coverslip

Table 3. Induction of MN by Colcemid (Continued)

B.

Colcemid (mg/ml)	Per Cent Cells with MN Termination Time			
	36 HR		48 HR	
	I	II	I	II
.0005	16	2	21	18
.0025	9	11	11	13
.0001	6	7	14	11
0	0	1	0	0

I = 6×10^3 cells/cm^2
II = 1.2×10^4 cells/cm^2

nuclei; yet even under these conditions, MN with kinetochore staining appeared present in the Colcemid-treated cultures. However, kinetochore staining in MN in X-ray treated cultures was apparently present also. Due to poor staining conditions, no attempt was made to quantify the frequencies of kinetochore-bearing MN in either X-ray- or Colcemid-treated cultures, however, it is believed that this approach is ultimately feasible.

6. CONCLUSIONS

It was found that kinetochore staining can be accomplished on a routine basis on mammalian interphase cells obtained from _in vitro_ and _in vivo_ material. However, the current FITC-dependent immunofluorescence technique does not appear to offer the staining intensity and/or persistence to allow for an accurate enumeration of the total genomic complement of a Chinese hamster, mouse, or human cell. This lack is primarily due to the three-dimensional structure of the interphase nucleus on the coverslips. Suggested future directions for further research in this area include: (i) the use of a cell line with a low diploid chromosome number, e.g., Indian Muntjac cells (n = 5,6) this would not be the best approach if there is an inverse relationship between nondisjunction frequency and chromosome number); (ii) technical manipulations to diminish cell thickness (there are probably technical manipulations other than hypotonic shock and differential centrifugation, which may be useful) and (iii) other staining techniques (e.g., immunoperoxidase, biotynilation).

Mammalian interphase somatic cells from _in vivo_ tissues can be examined for kinetochores. However, if the cells are not grown on coverslips, additional technical manipulations are required such as the use of poly-L-lysine-coated coverslips to increase cell adhesion. The exact conditions under which the

cells are attached (type of fixative, etc.) are cell-type-specific and require experimental analyzing to optimize conditions.

Kinetochore structures are not visible in mature sperm that are either untreated or increased in size by various manipulations to break down sulfhydryl bonds. Whether the absence of staining results from an antigenic difference in the kinetochore in mature sperm or a technical artifact remains to be elucidated.

Kinetochore structures can, most likely, be detected in micronuclei, suggesting an extremely important role for this assay system in identifying aneuploidy-inducing agents which do not destroy the kinetochore (antigenically speaking). Methods have been developed for making smears of mouse peripheral blood for use in the kinetochore staining technique. However, the absence of large numbers of micronucleated cells will probably limit the usefulness of this approach. Collection of micronuclei by cell sorting or differential centrifugation after destruction of erythrocytes, may provide a method for collecting appropriate quantities of MN for use in kinetochore evaluation.

It is suggested that these observations on kinetochore counting in interphase cells indicate the necessity of other technical developments and the initial observations on kinetochore staining in micronuclei suggest a possible research tool for the future.

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